AN ANALYSIS OF THE INITIAL REACTION IN THE SEQUENCE RESULTING IN HOMOLOGOUS SPLENOMEGALY IN THE CHICK EMBRYO

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The splenomegaly induced in the chick embryo by chorioallantoic or intracoelomic grafts of homologous adult spleen, or by intravenous inoculations of homologous spleen cells (Danchakoff, 1916; Murphy, 1916; Willier, 1924; Ebert, 1951; Simonsen, 1957; reviewed Ebert, 1958, 1959b) is thought to be the consequence of at least two sequential reactions, an initial graft-versus-host reaction (DeLanney and Ebert, in Ebert, 1957; Ebert and DeLanney, 1960; Simonsen, 1957; see also Billingham and Brent, 1957) followed by a tissue-specific growth reaction, granulocytic proliferation probably being stimulated by products resulting from the partial necrosis produced by the initial immune reaction (Ebert, 1951, 1954; DeLanney, Ebert, Coffman and Mun, 1962; see also Weiss, 1960). Evidence has been advanced also for the involvement of a third process, *i.e.*, a host-versus-graft reaction (Ebert and DeLanney, 1960; Ebert, 1961b); *cf.* Warner and Burnet, 1961), but it is not clear to what extent this reaction contributes to the splenomegaly. It is pertinent to inquire whether these processes can be separated experimentally.

The graft-versus-host phenomenon is but one manifestation of the familiar homograft reaction that leads to the rejection of tissue grafts; in several species, both cold- and warm-blooded, it has been shown to be a consistent and reproducible immunological reaction (Ebert and DeLanney, 1960). The immunological character of this first, destructive phase is widely accepted, being dictated by several lines of evidence.

(1) Recent findings in experiments using grafts of spleen from inbred lines of fowls have demonstrated that interstrain grafts produce a larger effect than intrastrain grafts, a finding to be expected if the reaction were an immunological one. Additional findings to be advanced here agree with those reported by Cock and Simonsen (1958), Mun, Kosin and Sato (1959), and Jaffe and Payne (1961) who used inbred strains of white Leghorn chickens.

(2) X-irradiation of a graft of adult chicken spleen removes its ability to affect the homologous organ of the embryo. According to Mun, Kosin and Sato (1959), after irradiation at low doses, splenic grafts retain their effectiveness; at moderate doses, a significant decrease in effectiveness is observed, and at high doses all activity is lost. Kryukova (1959) also showed that the inoculation of non-irradi-

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ated homologous spleen cells and cells x-irradiated at low doses caused approximately six-fold enlargement of the embryo spleen, yet cells irradiated with moderate doses had little or no effect. Other treatments, *e.g.,* boiling, and freezing and thawing, also remove all activity (Mun, Kosin and Sato, 1959).

(3) The effectiveness of a tissue in producing the reaction varies directly with its content of immunologically competent cells; hence, it appears consistent to state that the reaction is tissue-specific in the limited sense that the specificity reflects the proportion of competent cells a tissue contains. It may be premature to attempt to relate this specificity to a specific cell type, although Terasaki (1959) believes the large lymphocyte to be the common denominator. Whatever the nature of the cell (or cells) concerned, it is known that plasma cells, indicators of immune reactions, may be found at the terminal stages of the reaction (Mori, in Ebert, 1961a). All of the following tissues are effective: bone marrow, liver, spleen, and thymus (Danchakoff, 1918; Willier, 1924; Ebert, 1951). Moreover, Van Alten and Fennell (1959) and Billingham and Silvers (1959), respectively, have shown that a graft of either small intestine or skin also may affect the host's spleen. Although Ebert's earlier (1954, 1959b) observations of quantitative differences in effectiveness were based soundly, his generalization that one might expect a hierarchy of decreasing effectiveness, *e.g.,* spleen, thymus, liver, is difficult to establish. Solomon (1961) was unable to observe such differences and argued that the ultimate extent of the splenomegaly is related directly to the number of competent cells in the graft; certainly this figure will vary, although it is reasonable to assume that generally, spleen and thymus will have a larger number of such cells per unit volume or weight than will other tissues.

The reaction is class-specific; grafts of spleen of other avian species, such as duck, turkey, and pheasant, produce some effect but never as much as homologous spleen; rat, mouse and guinea pig spleens are completely ineffective in the chick (Ebert, 1951, 1954; Mun, Kosin and Sato, 1959). Presumably, the ineffectiveness of mammalian cells is a consequence of their failure to survive the rigors of the foreign environment long enough to produce an immune response. This explanation is being tested experimentally. If it proves to be correct, then mammalian immune mechanisms would appear to be unusually sensitive to the avian environment, for there is evidence for the survival of other kinds of mammalian cells in the chick embryo (*cf.* Clarkson and Karnofsky, in Ebert and DeLanney, 1960, p. 97).

(4) Finally, the ability of splenic grafts to affect the host varies with the age of the donor; grafts of spleen from embryonic donors have little or no effect, the effectiveness of grafts increasing when they are taken from progressively older donors up to several months after hatching. Additional data are presented herein, supplementing the comprehensive recent account by Solomon (1961). Although there are unexplained exceptions to the rule (*cf.* Ebert, 1951; Solomon, 1961, pp. 359–363), the effectiveness of grafts is related directly to their immunological maturity. Our perspective of the problem of maturation of the immune response has been broadened by the findings of Makinodan and Peterson (1962) who have observed that the relative antibody-forming capacity of spleen cells of mice varies with age from one week to 29 months. A rapid increase in activity was noted from one week to one month, one less rapid from one to 8 months. A gradual decrease was then observed from the peak at 8 months through an additional 21 months.

Accepting the argument that a part of the splenic enlargement following a graft of adult spleen encompasses immune reactions, we may take up next the site of these reactions. How many cells leave the graft and enter the extraembryonic membranes and the embryo itself? How many donor cells take up residence in the homologous organ of the host? Do they also settle in other organs? It is clear that when suspensions of adult chicken spleen cells or suspensions of adult chicken lymphocytes are administered to the embryo intravenously, or when grafts of adult spleen are made to the chorioallantoic membrane or into the coelom, *some* of the donor cells colonize the organs of the host. The evidence is derived from serial transfer studies by Simonsen (1957) and Ebert and associates (Ebert, 1957; DeLanney, Ebert, Coffman and Mun, 1962). When a graft of adult chicken spleen is made to the coelom of a four-day-old chick embryo, the host's spleen is enlarged four- to five-fold within six days. If fragments of this greatly enlarged ten-day-old embryonic spleen now are transferred to new four-day-old hosts, they elicit a reaction of the same order of magnitude, whereas fragments of spleen from normal ten-day-old embryos are ineffective. After nine successive transfers, the effectiveness of the implant is not reduced markedly below the level attained by the primary graft. Assuredly, then, there is some colonization. But how much, and to what extent do these donor cells proliferate? Simonsen (1957) argued that colonization and proliferation accounted for all the effects of splenic grafts. However, studies by Ebert and associates (summarized by DeLanney, Ebert, Coffman and Mun, 1962) of the cellular nature of the response pointed to the host as the principal source of proliferating cells. Moreover, studies using grafts radioactively labeled, in early experiments with sulfur^{35}, while not decisive, revealed a predominant localization of material in the homologous organ, but precluded a massive transfer of cells (Ebert, 1954, 1959b). Biggs and Payne (1959) have presented significant findings in a study in which they identified proliferating donor cells in chick embryos injected with adult chicken blood. In the chicken the fifth largest chromosome is paired in the male, unpaired in the female. Cockerel blood was injected into fourteen-day-old embryos which were sacrificed at day eighteen. In enlarged spleens taken from female embryos, male chromosomes could be identified, proving the localization of some donor cells. The relatively high number of dividing female cells, however, suggested to Biggs and Payne that an appreciable component of the splenic enlargement is provided by cells of the host. The evidence available, therefore, suggests that following the intravenous injection of blood or spleen cell suspensions, some donor cells colonize the host's spleen. Moreover, such colonization need not result invariably in splenic enlargement, which may result in whole or in large part from proliferation of cells of the host.

The fact that splenomegaly is not evoked by noncompetent homologous donor cells or with competent isologous cells forces the conclusion that the *proliferation of cells of the host is a secondary consequence of a primary immune reaction.* The nature of this secondary reaction must be the principal target of future investigations. In beginning such a study, it became clear that more information was needed on the extent of colonization and maintenance of donor cells in the several tissues of the host.

It is the objective of this report to present findings bearing on that question; these findings bear importantly also on another question, namely, the ability of the

embryonic environment to support an immune reaction. Preliminary accounts of some of these findings have been published (Mun, Errico and Ebert, 1961; Ebert, 1961a, 1961b).

Materials and Methods

Non-inbred white Leghorn chickens and eggs were supplied by Elder Farms, Hyde, Maryland. Hybrid white Leghorn chickens and eggs were obtained from Truslow Hatchery, Chestertown, Maryland. Chickens and eggs from two inbred lines (7 and 15) with coefficients of inbreeding of greater than 95% were supplied by B. Winton, Director of the Regional Poultry Research Laboratory, East Lansing, Michigan. New Hampshire red eggs were purchased from Red Gate Farm, Newport, New Hampshire. Eggs were incubated in a Jamesway incubator at 37.5 to 38.0° C.

The aseptic grafting technique employed was that described by Willier (1924; see also Hamburger, 1960). A quadrilateral window (1 × 1 cm.) was cut in the shell with a fine-toothed hacksaw blade. The shell membrane was punctured and reflected, and a fragment of tissue measuring approximately 1 × 1 × 2 mm., and weighing 5 to 10 mg., was placed on the chorioallantois. The shell membrane and shell were replaced and sealed with paraffin. The eggs were placed in the incubator with the small end down.

The eggs were operated on the ninth or tenth day of incubation. After 7 or 8 additional days of incubation the graft was removed and examined. The size of the area of implantation (length × width) was recorded and the condition of the implantation site was graded as follows: (1) graft enlarged, pink, and larger than the original; (2) graft pink and as large as the original implant; (3) graft brown or green, clearly not incorporated and smaller than the original or grafted tissue. Rarely a graft in category (3) produces a response, but the fact that a reaction did occur occasionally suggests the movement of viable cells from the graft soon after implantation. Spleens of recipient embryos were removed and weighed to the nearest 0.2 mg. The weights of spleens from embryos in group 3 were not included in the tabulations.

Rate of Colonization in the Chorioallantoic Membrane Adjacent to Spleen Grafts, and in the Hosts' Spleens

DeLanney and Ebert (1959a, 1959b), and DeLanney, Ebert, Coffman and Mun (1962) have followed the cytological changes in the chorioallantois at closely timed intervals after implantation of homologous adult spleen. Immediately after implantation the epithelium of contact thickens; the mesenchyme forms spindle cells and undergoes a shift toward myelogenesis. In the zone of contact between graft and membrane, the chorionic epithelium is eroded, clusters of granulocytes appear, spindle cells gather at the border, and tongues of cells, apparently originating in the graft, invade the membrane. The second set (or third set) chorioallantoic transplantation of fragments of chorioallantois taken from reactive sites surrounding the original first set spleen implant results in intensified reactions. Further evidence of colonization of the membrane is provided by the following experiments, in which fragments of homologous embryonic spleen were placed on the chorioallantois some distance from grafts of homologous adult spleen. After varying intervals, the embryonic grafts were removed, and their ability to produce splenomegaly determined.

Two windows, approximately 1 cm. apart, were cut in the shell. A fragment of adult spleen, kidney, or heart was placed on the chorioallantois through one window and 17-day-old embryo spleen was implanted through the other. In control groups embryonic spleen was implanted in both sites. After 7 additional days of incubation, the adult graft and the embryonic graft were removed, with associated membrane, and implanted on the chorioallantoic membrane of new 10-day-old hosts. After 7 additional days of incubation the hosts' spleens were removed

TABLE I

Colonization of embryonic spleen grafts adjacent to grafts of adult spleen and other tissues

Donor	No.	Mean weight of host spleen (mg.)	SE_m
Adult spleen + adult spleen	4	47.4	—
Graft of adult spleen	3	39.1	—
Graft of host's spleen	2	118.4	—
Adult spleen + embryo spleen	30	35.9	3.8
Graft of adult spleen	4	38.9	—
Graft of embryo spleen	29	40.3	4.9
Graft of host's spleen	2	36.1	—
Adult kidney + adult kidney	26	17.2	1.7
Graft of adult kidney	14	19.9	3.2
Graft of host's spleen	4	21.7	—
Adult kidney + embryo spleen	22	18.9	2.1
Graft of adult kidney	14	12.1	4.2
Graft of embryo spleen	19	38.8	6.6
Graft of host's spleen	4	42.3	—
Adult heart + adult heart	9	12.2	0.8
Graft of adult heart	6	18.4	4.9
Graft of host's spleen	6	18.7	4.4
Adult heart + embryo spleen	12	14.9	1.2
Graft of adult heart	3	14.2	—
Graft of embryo spleen	10	16.6	1.6
Graft of host's spleen	9	20.5	3.5
Embryo spleen + embryo spleen	10	13.7	0.9
Graft of embryo spleen	8	12.4	1.0
Graft of host's spleen	3	14.6	—

and weighed. Table I shows that an embryonic spleen graft placed adjacent to an adult spleen graft can affect the host's spleen to the same extent as an adult spleen graft. Here, then, is further evidence of movement of cells from the adult spleen graft to a graft of embryonic spleen on the membrane. How rapid is this movement?

Homologous spleen and homologous embryonic spleen were implanted approximately 1 cm. apart as described above. After 2, 3, 5, 6, or 7 additional days of incubation both grafts and the host's spleen were removed and transferred to new

10-day hosts. Table II shows that, as early as *two* days after implantation, both the embryonic spleen graft and the host's spleen, neither of which show any enlargement at this time (*cf.* DeLanney, Ebert, Coffman and Mun, 1962), are capable of affecting the spleen after serial transfer.

Colonization of Adjacent Membrane by Adult Spleen Cells Labeled with Tritiated Thymidine

The suggestion that the use of cells labeled with tritiated thymidine might aid in resolving the question of migration of adult chicken spleen cells from grafts to the hosts' membranes and spleens was advanced by Ebert (1959b). However, the principal limitation of the method, the dilution of label in rapidly dividing cells, is critical, and in the opinion of the writers the technique is less reliable than the cytological method, *i.e.*, recognizing donor cells by sex chromosome differences (Biggs and Payne, 1959; Ohno, 1961). However, the following experiments do

Table II

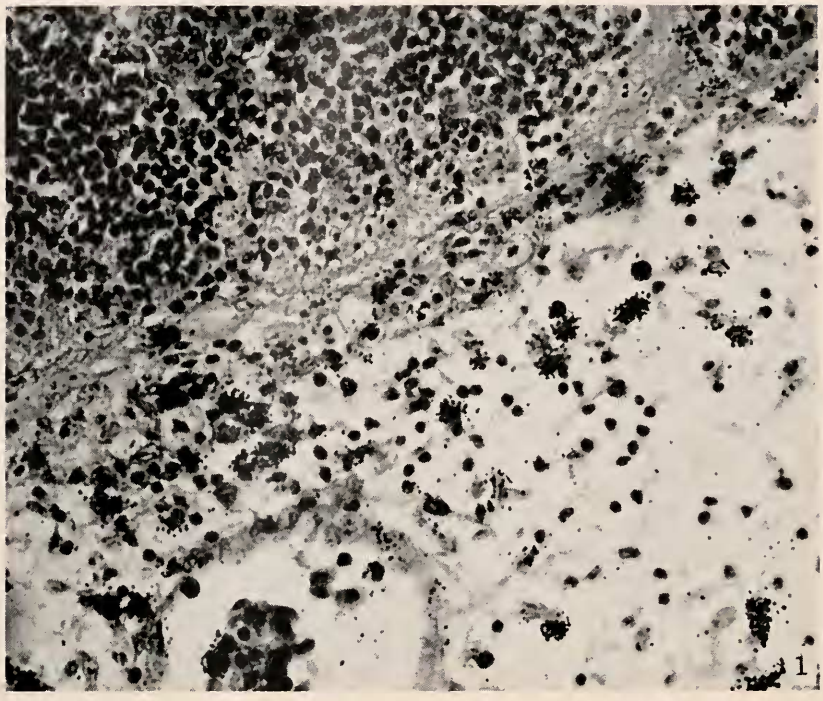
Rate of colonization of host embryo's spleen (HS) and embryonic spleen graft (GES) adjacent to adult chicken spleen graft (GAS) on the chorioallantois

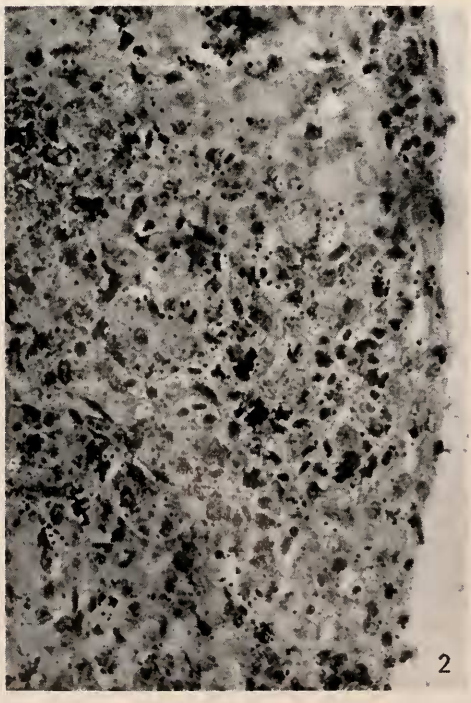
Donor	2–3 days			5 days			7–8 days		
	No.	Mean weight of host's spleen (mg.)	SE_m	No.	Mean weight of host's spleen (mg.)	SE_m	No.	Mean weight of host's spleen (mg.)	SE_m
AS + ES									
GAS	13	21.6	6.8	2	47.8	—	4	38.9	—
GES	21	21.8	3.4	5	21.8	6.3	29	40.3	4.9
HS	14	23.1	5.1	6	17.5	2.0	30	35.9	3.8
ES + ES									
GES	6	10.8	1.2				8	12.4	1.0
HS	4	11.4	—				3	14.6	—

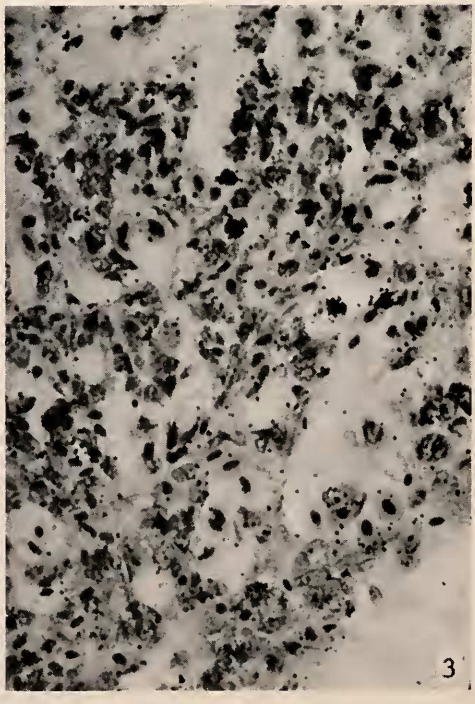
contribute further to our knowledge of the migration of cells into the adjacent membranes.

Nineteen experiments were conducted using labeled and non-labeled donor material. The results of three experiments (XI, XIII, XIIIa) will be considered here.

In experiment XI, the donor tissues were labeled by injecting into the wing veins of adult white Leghorn chickens 2 millicuries of tritiated thymidine in two doses, 48 and 24 hours before sacrificing. In experiments XIII and XIIIa, labeled tissue from enlarged embryonic spleen was used as donor tissue. The cells were labeled by injecting 15 to 25 microcuries of tritiated thymidine into the yolk sacs of 9-day-old chick embryos. Twenty-four hours later, a piece of unlabeled adult chicken spleen was implanted on the chorioallantois of each embryo (Fig. 1). In control series, a piece of homologous embryo spleen was implanted instead of adult spleen. After 7 or 8 additional days of incubation the enlarged







FIGURES 1–3.

and labeled host spleens were removed, cut into small pieces, and implanted in 9-day-old chick embryos.

The latter approach resulted in at least a doubling of the percentage of labeled donor spleen cells. The enlarged embryo spleen also elicited a greater increase in the size of the host spleen than grafts of adult chicken spleen. Large white nodules and lesions were observed more frequently in foot and head regions.

At recovery a number of tissues, including the membrane containing the graft, the host's spleen, and a sample of blood, were obtained from each embryo. Representative whole embryos also were fixed in Bouin's fluid.

The labeled cells were detected by autoradiography. The host's spleen and membrane containing the graft were sectioned at 5 microns, and stained with Mayer's hematoxylin and eosin. The slides were then coated with Kodak NTB-3 photographic emulsion, following in general the procedures developed by Messier and LeBlond (1957) and Everett and Simmons (1953). Approximately three drops of a 50% emulsion kept at 40° C. were smeared on the surface of the warmed glass slide with a wet brush. The smear was slowly rocked to remove the brush marks. Excess emulsion was shaken off, and the slide was permitted to dry in a near-vertical position. The coated slides were kept in the refrigerator (4 to 10° C.) and developed in D72 or D19 (Kodak) after 14 days.

In each experiment, more than 30 embryos received grafts of adult chicken spleen or enlarged embryo spleen on the ninth day of incubation. An equal number received labeled embryonic spleen, labeled adult chicken kidney, or irradiated and labeled adult spleen grafts. The different categories of active and inactive, as well as labeled and nonlabeled donor tissue, also were combined on the membrane of the same host. A small number was untreated or received three drops of saline.

A number of embryos from each group, selected at random, were recovered at postoperative days 1, 2, 3, and 7. Another group, sufficient in numbers to ascertain the degree of enlargement of the host's spleen elicited by the donor material, was recovered on the eighth day after the operation (Table III).

In embryos in which donor spleen tissues containing distinctly labeled cells (see Figure 2) were grafted, labeled cells were still detected in significant numbers in the graft as well as the adjacent membranes up to the fifth postoperative day (Fig. 3). By the eighth postoperative day, however, labeled cells were not readily detected in the graft and adjacent tissues. Preliminary examination of spleens from host embryos, which contained distinctly labeled donor spleen cells in the CAM graft, revealed few or no distinctly labeled cells. Quantitative evaluation of the autoradiograms is in progress; however, these preliminary observations do not suggest a direct large scale migration of donor cells from the CAM graft to the host's spleen.

FIGURE 1. Chorioallantoic membrane showing the edge of a graft of adult chicken spleen. Chick embryo injected via the yolk sac with 25 microcuries of tritiated thymidine, and the unlabeled graft implanted on the ninth day of incubation; recovered 24 hours later. ×500.

FIGURE 2. Section of spleen from an embryo which received 25 microcuries of tritiated thymidine and a graft of adult chicken spleen on the ninth day of incubation; recovered after 8 days. ×500.

FIGURE 3. Section of chorioallantoic membrane of a 13-day embryo containing cells of a labeled graft of "second-set" embryonic spleen, implanted 4 days earlier. ×500.

TABLE III

Mean weight of host's spleen after chorioallantoic grafting of adult and embryonic spleen

Expt. (see text)	Day recovered post-operative	Donor	No.	Average weight of host spleen	SE_m
XI	8	Adult chicken spleen	5	34.0	2.6***
		Saline control	7	10.5	0.6
XIII	8	Enlarged embryo spleen	10	86.2	8.3***
		Saline	5	12.4	1.4
$XIII_a$	8	Enlarged embryo spleen	7	29.4	4.5**
		Saline	4	8.8	1.0

** Significant at the .01 level.
*** Significant at the .001 level (*t* test).

COLONIZATION IN OTHER ORGANS

It might be expected that competent cells from a graft might be lodged in all tissues of the embryo's body, to some extent, possibly to the same extent to which the adult tissues normally contain lymphoid and other reticulo-endothelial elements. This expectation is realized.

Adult chicken spleen was implanted on the chorioallantois of 9-day host embryos. After 7 more days of incubation the host's spleen, liver, heart, and kidney were removed and implanted on membranes of new 9-day recipients. Table IV shows an increase in the weights of such secondary hosts' spleens receiving grafts of "second set" spleen, liver, heart, and kidney, to approximately the same extent as that elicited by the corresponding adult organs.

Thus there is a transfer of competent cells not only to the host's spleen but to other organs as well.

SERIAL PROPAGATION IN CHICK EMBRYOS OF EMBRYONIC SPLEEN CELLS FROM NON-INBRED AND INBRED CHICKENS

The graft-versus-host reaction has provided unequivocal evidence that, beginning as early as the fourth day of development, the chick embryo provides an

TABLE IV

Effect of grafts of fragments of adult organs and organs from embryos stimulated by seven days' exposure to adult spleen grafts on the weight of the host embryo's spleen

Nature of grafts	Spleen weight (mg.) following grafts of			
	Spleen	Liver	Heart	Kidney
Adult organs	47.4 (4)*	10.8 (7)	18.7 (6)	17.2 (26)
Host embryo organs after grafting of adult spleen	42.7 (6)	18.0 (6)	12.1 (7)	23.6 (6)
Embryonic organs	14.6 (3)	11.4 (5)	12.3	8.0
Host embryo organs after implantation of embryonic spleen on the membrane		16.0 (3)	11.9 (3)	12.4 (3)

* Figures in parentheses indicate number of cases.

environment favorable for at least one class of immune reactions, those of transplantation immunity (Ebert, 1961b). Competent cells retain their competence in the embryonic environment. But would incompetent embryonic spleen cells which were maintained in an embryonic environment for long periods of time ever reach a stage of functional maturity? Or, to put the question in more practical terms, if homologous embryonic cells were transferred from the graft to the host's spleen where they proliferated, after several transfers the donor embryo cells thus maintained in this embryonic environment should eventually attain maturity and be able to elicit an enlargement of the host spleen. On the other hand, if the

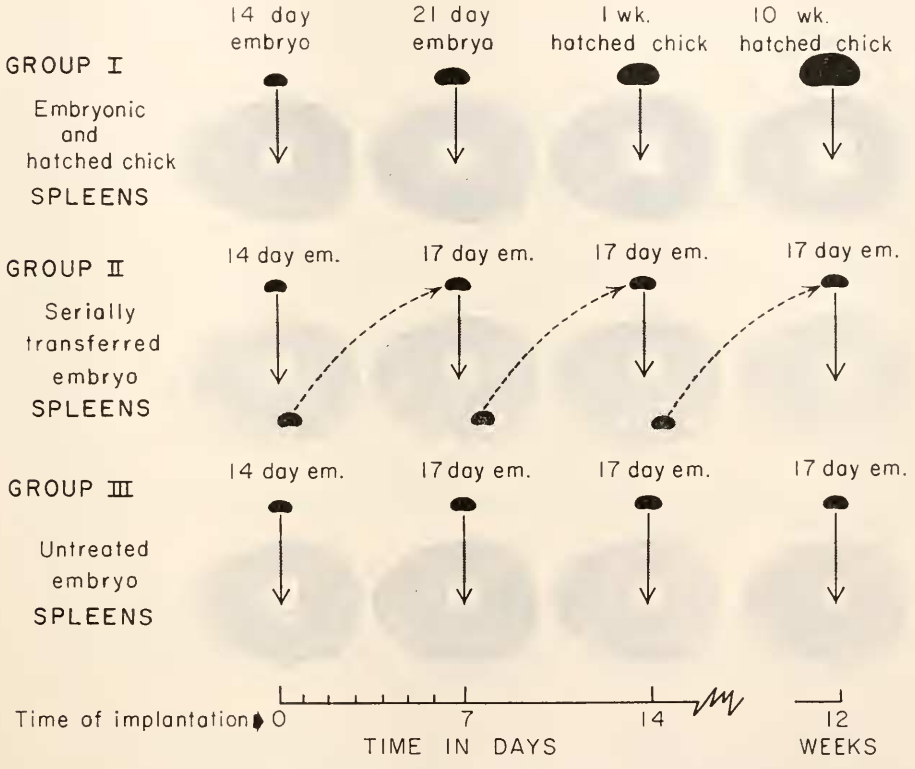


FIGURE 4. Serial grafting of homologous embryonic spleen.

donor embryo cells were incorporated in the host's spleen but did not proliferate, they would be diluted after only a few passages.

Four groups of embryos have been studied: (1) Fragments of homologous spleen from successively older embryos and hatched chicks were grafted to the membranes of 10-day-old chick embryos at weekly intervals. After 7 additional days of incubation, the hosts' spleens were removed and weighed to the nearest 0.2 mg.

(2) A fragment of homologous 14-day embryonic spleen was placed on the membrane of a 10-day host embryo. After 7 additional days of incubation, the host's spleen was removed, weighed, and transferred to another 10-day embryo.

This procedure was repeated for 7 or 11 weeks. The donor spleens were not pooled but were kept separate. Thus, for each of the 20 initial donors 20 separate lines may be traced.

(3) Fourteen- or 17-day-old embryo spleens were placed on the membranes of 10-day host embryos at weekly intervals. After 7 additional days of incubation the hosts' spleens were removed and weighed.

These three groups are illustrated graphically in Figure 4.

(4) The weights of the 14- or 17-day embryo donor spleens before grafting comprise the fourth group. This group is not included in the final tabulation because greater variation in spleen weight was observed following implantation

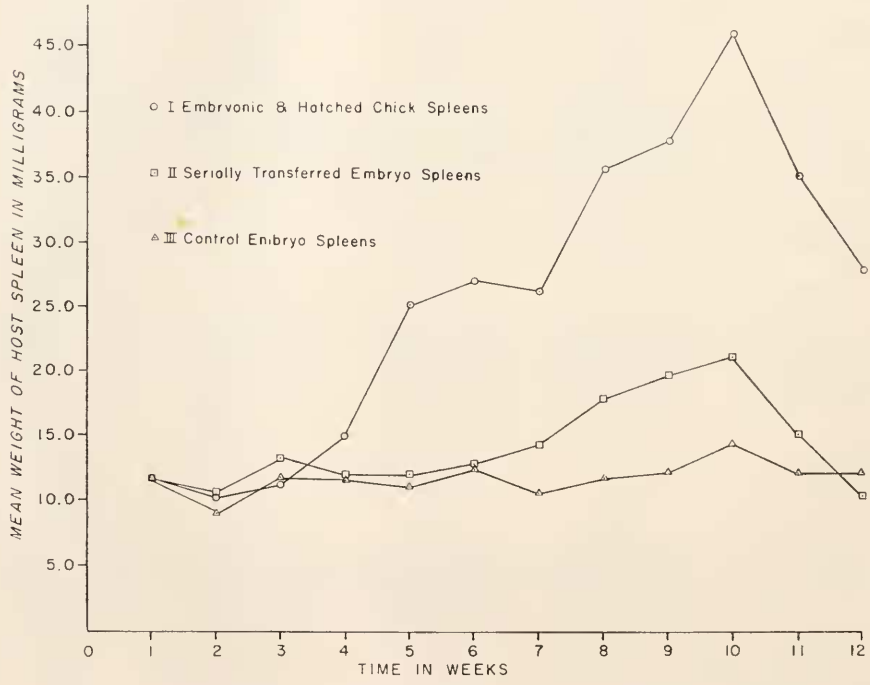


FIGURE 5. Splenomegaly after serial grafting of homologous embryonic spleen.

of a piece of embryo spleen. Thus the spleen weights in the third group were better controls for the second group.

The first experiment was carried out over an 8-week period. Because an increase in the average weight of the host's spleen in the serial group (2) was observed in the eighth week, the second experiment was carried out over a longer 12-week period.

The results of the two experiments were consistent, hence the data were pooled.

As may be seen in Figure 5, spleen grafts from successively older embryos and hatched chicks after a short lag period of one or two weeks produce a progressively greater enlargement of the host chick embryo spleen. A significant enlargement of the host embryo spleen is produced by spleen from a 3-week-old hatched chick.

These findings are in general agreement with those of Solomon (1961) and the previously unpublished data of DeLanney (cited in Solomon, 1961) who observed an approximate doubling in spleen size following grafts of spleen from 28-day-old juvenile chickens. DeLanney's independent findings are not strictly comparable to those set forth here, the period of exposure (days 7 through 18) and weighing procedure being different, hence they are not included in the tabulation; the data may be obtained from him upon request.

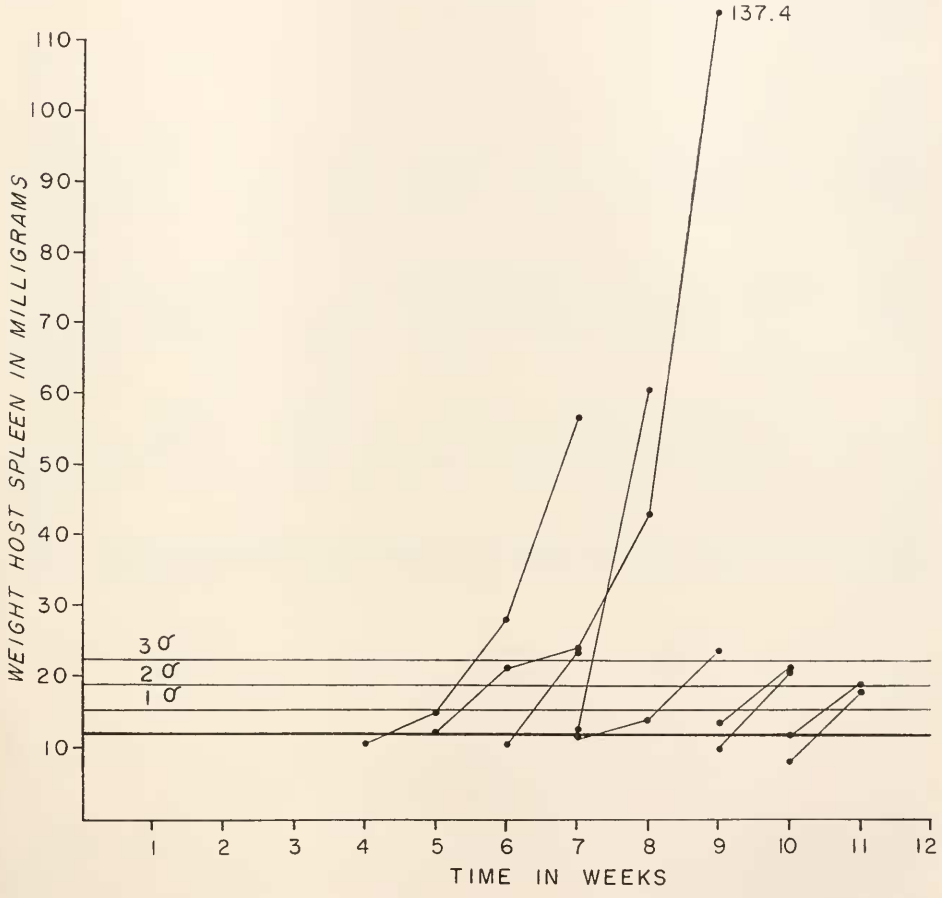


FIGURE 6. Splenomegaly in individual lines after serial grafting of homologous embryonic spleen.

In the second or serial group, after a lag period of 6 to 7 weeks, a distinct increase is observed in the average weight of the host's spleen. However, the differences in the mean weight between the serial group and the third, control, group even at the peak of 8 weeks is significant only at the 5% level as determined by Students "*t*" test.

However, when one follows the changes in spleen weights of subsequent hosts in each of the individual lines in the serial group, a clearer picture emerges. Of

the 30 initial donors only 14 lines were successfully transferred for 8 to 12 weeks. Figure 6 shows fragments of 9 of these 14 surviving lines. The lag period of 6 to 8 weeks is not shown. In 4 of these 14 lines, after the lag period, there is a definite progressive increase in the weights of the hosts' spleens. Five other lines show this increase to a lesser extent and 4 lines do not show any change in weight of the hosts' spleens.

The individual weights of many of the host spleens at the peak of the growth phase deviate greatly from the distribution of the control (group 3). The 3 s level (97%) for group 3 is shown (22.3 mg.).

The data show that in 4 out of 14 lines the effects are cumulative, *i.e.*, an increase in spleen weight occurs with each successive transfer. We may interpret these observations as indicating that in these 4 lines, cells of donor origin are transferred from the graft to the host's spleen. It would appear that these embryonic spleen cells proliferate and are maintained in the host embryonic environment. After several transfers, following the pattern of development of the normal chick spleen, the cells mature immunologically. The splenomegaly thus induced is serially propagated.

One may ask next, are the cells which produce this effect truly derived from the *first,* or do they stem from the second, or any of the subsequent transferred spleens? Is a single initial exposure to antigen sufficient to produce splenomegaly in subsequent hosts of a series? At least a tentative answer to this question is obtained by the use of embryos from two inbred lines of chicks.

Mun, Kosin and Sato (1959), using two inbred lines of chickens, found that a greater splenomegaly was obtained when the donor tissue was derived from an adult chicken of the opposite line than from the same line. Cock and Simonsen (1958) made similar observations using injection techniques. It should be possible to determine if a single exposure is sufficient by the following experiment, illustrated diagrammatically. One need only compare the effectiveness of the two series:

(1) $A \rightarrow B \rightarrow B \rightarrow B \rightarrow B \rightarrow B \rightarrow B$ and (2) $B \rightarrow B \rightarrow B \rightarrow B \rightarrow B \rightarrow B \rightarrow B$,

where A is the donor spleen from one line and B is the other line.

The effects of spleens from two inbred lines were first compared within and between lines. Spleen tissue from one-month-old line 7 and line 15 chickens were implanted, reciprocally and within lines to the membranes of 10-day-old embryos. As shown in Table V there is a striking difference in the reaction of these two lines to line 7 donor spleen but not to line 15 donor spleen. However, the line 7 embryo spleen was affected somewhat more greatly by line 15 donor spleen than by line 7 donor spleen. These differences are significant on the basis of the pooled *t* test.

The serial experiment as outlined above was then carried out on embryos from these two inbred lines. Four groups of embryos were treated as follows:

(1) A 17-day line 7 embryo spleen was placed on the membrane of a 10-day line 15 embryo. After 7 additional days of incubation, the host's spleen was removed, weighed to the nearest 0.2 mg. and cut in half. One half of the host's spleen was transferred to the membrane of another line 15 embryo. After 7 days the host's spleen was again removed, weighed, and treated in similar fashion. The average weight of the line 15 hosts' spleens forms the first group (I) in Table VI.

TABLE V

Comparison of the ability of spleens from two inbred lines of chickens to affect spleens of embryos from these two lines

Line of adult donor	Line of host embryo	No.	Average weight of host's spleen (mg.)	S.E. of mean
7 (4 donors)	7	28	13.0	0.5
	15	17	75.5	8.4
15 (3 donors)	7	22	29.3	1.8
	15	17	25.2	2.8

(2) The other half of the line 15 embryo spleen was placed on the membrane of a line 7 embryo. After 7 additional days of incubation, the host's spleen was removed and weighed, but not transferred. The average weight of the line 7 hosts' spleens form the second group (II).

(3) In the third group, a 17-day line 15 embryo spleen was grafted to a 10-day embryo from the same line. After 7 additional days of incubation, the host's spleen was removed, weighed, and cut in half, one half being transferred to a new host of the same line. The average weight of the line 15 hosts' spleens forms the third (III) group.

(4) The other half of the line 15 spleen was grafted to a line 7 embryo. After 7 additional days of incubation, the spleen was removed and weighed but not transferred. The average weight of the line 7 hosts' spleens forms the fourth (IV) group.

(5) As further controls, untreated 17-day-old lines 7 and 15 embryo spleens were grafted on line 7 embryos each week. The average weight of the donors' and hosts' spleens formed a fifth (V) group. The data for this group are not included in the table.

As shown in Table VI a significant increase in the weights of the hosts' spleens was not observed in any group after 8 transfers. These results suggest that a

TABLE VI

Mean weight of spleens of four groups of inbred embryos serially transferred at weekly intervals

Week number	Group I	Group II	Group III	Group IV
1	10.2 (18)*		10.1 (18)	
2	11.4 (11)	9.3 (10)	13.9 (13)	10.1 (12)
3	13.3 (15)	10.2 (5)	13.8 (17)	10.7 (9)
4	13.4 (14)	10.5 (10)	13.2 (12)	10.4 (12)
5	13.5 (16)	9.9 (11)	12.4 (15)	10.0 (11)
6	13.8 (14)	11.0 (12)	13.6 (10)	10.0 (8)
7	13.4 (15)	9.4 (12)	14.5 (13)	10.5 (7)
8	14.4 (18)	7.8 (12)	13.5 (17)	9.3 (8)
9	14.9 (15)	8.2 (11)	13.2 (12)	10.8 (11)

* Figures in parentheses indicate number of cases.

single exposure (A → B) was not sufficient to initiate the reaction. In view of the observation that a subsequent increase in weight of the hosts' spleens was obtained in serial transfers of spleens from non-inbred embryos, it must be suggested that the latter effect is cumulative. Homologous cells of different genetic makeup are accumulated gradually in the spleen with each transfer, resulting eventually in the observed reaction.

However, in view of the fact that the homologous hosts and donors involved were all embryonic, why was a mutual immunological tolerance not developed? We were led to inquire then whether "tolerance," as measured by the prevention of splenomegaly, could be induced by the exposure of 10- to 17-day-old chick embryos to grafts of embryonic spleen?

Effect of Spleens From Adult New Hampshire Red Chickens Which Had Received Chorioallantoic Grafts of White Leghorn Embryo Spleen on the Ninth Day of Incubation

Terasaki, Cannon and Longmire (1958) injected 0.4 ml. of blood intravenously from 10- to 16-day-old white Leghorn (WL) embryos to New Hampshire red (NH) embryos and vice versa. Two or 15 days after hatching, skin from chicks other than the blood donor, but of the same breed as the blood donor, was grafted. A significant percentage of these homografts survived longer than grafts between control chicks not previously injected with blood. This observation was extended to include interbreed differences by Kulangara, Cannon and Longmire (1959). Tolerance of skin homografts may be obtained by embryonic injection of blood from a breed of chicken other than that of the skin donor. The following series of experiments was designed to answer the question, can embryos of one breed (NH) be made "tolerant" with respect to the ability to affect the spleen of another breed (WL)?

Embryo spleens pooled from five 19-day-old white Leghorn embryos were minced and pipetted on the membranes of 10-day New Hampshire red hosts. The operated eggs were permitted to hatch. On the second, third, and tenth week post-hatching, spleens from treated and untreated chickens were implanted on the membranes of 10-day-old WL hosts. The results are shown in Table VII.

Table VII

Effect of spleens from 2-, 3-, and 10-week-old New Hampshire red (NH) chickens grafted with white Leghorn embryo spleens (WL-ES) on the 10th day of incubation

Donor	No.	Mean weight of host's spleen (mg.)	SE_m
2-week-old NH + WL-ES	21	16.1	1.5
NH not treated	24	16.4	3.9
3-week-old NH + WL-ES	24	14.8	1.2
NH not treated	12	16.4	1.9
10-week-old NH + WL-ES	31	32.2	3.7
NH not treated	28	28.2	3.5

The effect on the host spleen of 2- to 3-week-old chicken spleen is not large (see Figure 5) but there does not appear to be any difference between the groups. Spleens from 10-week-old chickens produce a four-fold enlargement. Again, there does not appear to be any difference in the ability of the spleens from treated and untreated chickens to elicit splenomegaly. Under the conditions employed, therefore, tolerance is not induced. Possibly the relative ineffectiveness of the membrane implantation technique, in contrast to intravenous injection, is to be stressed. In any event, there are insufficient grounds here for questioning the idea of interbreed tolerance in chickens.

Reduction in Effectiveness of Adult Spleen Following Pre-immunization

The availability of inbred lines of chickens made it possible to test further the possibility of pre-immunizing adult chickens and producing unusually rapid and severe graft-versus-host reactions. Earlier attempts with non-inbred fowls (Mun, Kosin and Sato, 1959; Van Alten, 1961) had produced anomalous results. The specific question to be answered is the following: will spleens from adult chickens of one line which have rejected skin grafts from another line produce a greater effect in hosts of the donor line than spleens from animals which had not previously rejected such skin grafts?

Skin grafts were performed on 10-day-old hatched chicks from inbred lines (7 and 15), both between and within these two lines. After one month, a great majority of the skin grafts received from the opposite line (homografts) began to disintegrate and slough, leaving large open wounds at the site of the graft. Three chickens (two from line 7 and one from line 15) showing the graft rejection reaction were sacrificed and fragments of their spleens were implanted on the membranes of 10-day-old line 7 and line 15 embryos. As controls, chickens with intact skin grafts from chicks from the same line (isografts), as well as autografts, and untreated chickens from each line were sacrificed at the same time. The results of these chorioallantoic grafts are shown in Table VIII. Spleen implants from chicks showing the graft rejection reaction did not elicit a greater enlargement in the reciprocal line host than spleen implants from the control chicks. In fact, the average weight of the host spleen was somewhat less than that of the control group in both lines.

These observations are similar to those reported by Mun, Kosin and Sato (1959) and Van Alten (1961). In the former experiments, adult chickens were injected intravenously and intraperitoneally with pooled 15- to 19-day-old chick embryo spleens. The spleens from these injected chickens did not produce a greater increase in the size of the host embryo spleen. Instead, the effect of spleen from the injected chickens was consistently and significantly less than that of spleens from non-injected chickens. Terasaki (1959) made similar observations. Donor chicks were immunized by skin grafting or by injection of spleen cells intravenously and intraperitoneally. Neither lymphocytes nor spleen cells from these immunized chickens when injected into embryos isologous with the immunizing tissue produced marked splenic enlargement or earlier deaths.

Simonsen and Jensen (1959) observed a marked graft-versus-host reaction (higher spleen indices) in the hybrid mouse $(C_3H \times AKR)F_1$ host when the

TABLE VIII

Effect of spleens from inbred WL adult chickens which had rejected skin grafts from the opposite line

Line of donor	Treatment of donor and condition of graft	Line 7 host			Line 15 host		
		No.	Mean weight of host's spleen	SE_m	No.	Mean weight of host's spleen	SE_m
7	(B3) rejected skin graft from line 15	5	12.2	13.3	9	77.1	13.2
7	(B8) rejected skin graft from line 15	4	19.5	3.9	9	79.0	18.2
7	(B4) autograph surviving	3	14.5	1.6	8	133.2	24.5
7	(B19) not operated	5	11.4	1.2	4	55.2	16.6
15	(Y9) rejected skin graft from line 7	12	19.2	2.2	3	47.3	—
15	(Y2) skin graft from line 7	10	36.1	6.9	2	13.1	—
15	(Y24) not treated	9	27.0	4.8	3	17.3	—

donor (AKR) was previously immunized with the hybrid cells. The failure to obtain similar results in the chicken may be due to an insufficient amount of homogeneity in the two lines used. In preliminary studies, 50% of skin grafts performed at 10 days post-hatching persisted for at least 5 months in line 7 hosts and for at least one to two months in line 15 hosts. Studies of the effects of spleens from chickens which have rejected a number of skin grafts from several different donors from the opposite line are in progress.

DISCUSSION

The extensive investigations of many laboratories, including our own, have led to the conclusion that the embryonic splenomegaly induced by grafts or injections of homologous spleen cells involves at least two major steps: donor cells actively pervade the host's reticulo-endothelial tissues, there to proliferate and mount an immunologic reaction against the host. The pattern of this attack in the host's chorioallantoic membrane and spleen has been followed by DeLanney and Ebert (1959a, 1959b; see also DeLanney, Ebert, Coffman and Mun, 1962). In spleens of hosts receiving grafts of adult chicken spleen, a pronounced shift toward granulopoiesis is observed by the eleventh day, followed by accumulation of mucopolysaccharide, breakdown of the vascular bed, and necrotic and fibrotic foci.

Biggs and Payne (1961) observed similar pathologic changes in the host spleen following inoculation of chick embryos with competent adult cells: extensive proliferation of reticulum cell foci, and the formation of blast cells and granulocytes. This phase is followed by a lymphoid transformation of the reticulum cell foci.

These observations and others suggest that although some of the cells initially transferred from the donor graft to the host spleen proliferate, cells of the host

proliferate also. Biggs and Payne observed that mitotic figures of both donor and host origin were present approximately in the proportion of 1:1 in spleens enlarged five- to twenty-fold. From cytological studies of changes in the host's spleen following inoculation of adult chicken blood, they argue that the reticulum cell foci, together with some of the blast cells, are of donor origin and that the majority of blast cells and developing granulocytes are of host origin, a conclusion in good agreement with the observations of the authors (Ebert, 1959; DeLanney, Ebert, Coffman and Mun, 1962). Although his earlier writings emphasized the proliferation of donor cells, Burnet (Burnet and Burnet, 1961; Warner and Burnet, 1961) now agrees that much of the proliferation is of cells of the host.

Most of the observations presented in the foregoing pages bear directly on the first phase of the reaction, lending support to the general argument advanced for its immunologic nature. For these, further discussion would be redundant. However, a few of the findings depart sufficiently from the expected to open new questions for discussion.

Earlier, one of us (Ebert, 1961b) had argued that the serial transfer experiments, using embryos of non-inbred lines, supported the idea that not only was the embryonic environment capable of supporting immune reactions, but also that a line of cells derived from the very first graft generation matured immunologically in that environment. Although the experiments reported herein with inbred lines do not require a major change in that view, it is necessary to state that it is not possible to argue for the derivation of the effective cells from the initial graft. What appears to be necessary is the accumulation of a threshold number of homologous cells; under the grafting conditions employed, one transfer is insufficient (*cf.* Howard and Michie, 1962).

Although we recognize that tolerance can be induced in adult animals (Rubin, 1959; Shapiro, Martinez, Smith and Good, 1961), it seems unlikely that the anomalous reduction in effectiveness of spleen taken from pre-immunized animals could be a degree of unresponsiveness as a consequence of competent cells introduced into an excess of antigen. Possibly, as an alternative explanation, the concept of allergic death (Boyse, 1959; Gorer and Boyse, 1959) may be advanced. Pre-immunized cells, exposed to antigen, if not immediately after implantation, at least upon invading the host tissues, undergo hyperactivity, resulting in their death. A test of this idea would be the examination of spleens of host embryos at time intervals after grafting shorter than the usual 5 days; if this argument were correct, one would expect a burst of donor cell proliferation, with early death of these lines.

Finally, we may comment briefly on the nature of the host's reaction. It is necessary to revive one of the several explanations which Billingham (1959) described as "ingenious" (p. 951). We do not believe that the development of the graft-versus-host concept has provided the "final solution" to the problem of homologous splenomegaly. The emphasis on donor cell proliferation (Billingham, 1959; Burnet and Burnet, 1960; Simonsen, 1957) resulted in a lack of interest in the host's response (Ebert, 1951, *et seq.*, reviewed 1959b; DeLanney, Ebert, Coffman and Mun, 1962). Earlier we advanced preliminary findings which suggested an incomplete immune reaction on the part of the host (Ebert and DeLanney, 1960; DeLanney, Ebert, Coffman and Mun, 1962). Although we

have no reason to doubt that argument, it has become increasingly clear that it is not a *sufficient* explanation.

Undoubtedly, the sum total of evidence requires that the first step of the reaction be an immune graft-versus-host reaction. This results in the initiation of the second step, an intense proliferation of host cells due to the release of growth-promoting substances from the immunologically damaged host cells. That damage, irrespective of the mechanism by which it is produced, can lead to growth promotion is now a well-established fact (Abercrombie, 1957; Argyris and Argyris, 1959, 1962; Bullough and Laurence, 1960).

Concomitantly with host cell hyperplasia the donor cells also continue to proliferate due to the host antigenic stimulus. With increase in the number of donor cells, a more intense immune attack on the host occurs, leading in turn to further damage, and to further proliferation of host cells. It is apparent that these two interactions will result in massive growth of the spleen. The relative contribution of host and donor cells to splenomegaly will vary, and we would expect a greater contribution from host cells since they are present in much larger numbers. Thus the wide variations in the relative contributions of host and donor cells to splenomegaly experimentally observed become understandable, and, in fact, expected.

This hypothesis helps us to understand another feature of splenomegaly which so far has remained unexplained, that of fibrosis and its associated metachromasia (Ebert and DeLanney, 1960). Connective tissue proliferation is to be expected after damage of an organ, along with parenchymal proliferation, since connective tissue is stimulated by damage just as parenchymal tissue is (Abercrombie, 1957). In addition, such connective tissue proliferation is usually associated with increases in mucopolysaccharides which are responsible for the intense metachromasia (Washburn, 1960). We do not know if the stimulation of connective tissue proliferation is due to relatively nonspecific growth-promoting substances released by damage (Abercrombie, 1957; Swann, 1958), or whether the graft directs a specific antibody attack on the connective tissue cells which in turn release tissue-specific growth-promoting substances. That growth-promoting substances released by damage might be tissue-specific is suggested by the recent work of Argyris and Argyris (1962), and Bullough and Laurence (1960).

The actual mechanism of growth promotion leading to splenomegaly is unknown, but it is related clearly to the mechanism advanced by Ebert (1951, 1954), which was in turn related to Weiss's template-antitemplate theory of growth regulation. According to this view (reviewed, Weiss, 1960), the introduction of disintegrating cells should release specific templates which would "combine with, or otherwise trap, homologous antitemplates, their presence in the pool will entail a temporary lowering of antitemplate concentration, hence again a spurt of growth in the homologous cell strains of the host" (p. 65). Or templates might be incorporated directly into homologous cells, accelerating the growth rate. Partial necrosis of an organ (which is precisely what is observed as a consequence of the graft-versus-host reaction) will have the same effect as partial removal, *i.e.*, compensatory growth. Hence the stimulating effects of tissue-specific ribonucleoprotein fractions (Ebert and DeLanney, 1960; DeLanney, Ebert, Coffman and Mun, 1962) and other lines of evidence (reviewed, Ebert and Wilt, 1960) must be re-examined in this light.

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Summary

1. As demonstrated by their capacity to induce splenomegaly and by tritium-thymidine labeling, some of the cells of chorioallantoic grafts of adult chicken spleen colonized the chorioallantois, spleen, and other organs of the host embryo within two days.

2. The capacity of the embryonic environment not only to support immune reactions but also to permit maturation of mechanisms of immune response was demonstrated by the serial propagation of embryonic spleen cells in non-inbred embryos. A cumulative response was obtained, beginning with the fifth or sixth transfer, approximately paralleling the normal development in the chicken of the ability to elicit splenomegaly.

3. However, stimulation of the host spleen was not obtained by the serial propagation of embryonic spleen cells in inbred embryos nor in a series in which the single initial donor was derived from a different inbred line. This suggested that the accumulation of a threshold number of reactive cells is necessary for the stimulation.

4. Induction of mutual interbreed "tolerance," as indicated by reduced effectiveness of adult chicken spleen to induce splenomegaly, was not obtained by previous chorioallantoic grafts of embryonic spleen.

5. The pre-immunization of adult chickens of one inbred line by skin homografts from a second line did not render the former's spleen capable of an enhanced reaction but, instead, reduced its effectiveness to elicit host spleen enlargement. It was suggested that such hyperimmunized cells undergo allergic death.

6. Attention is redirected to the proliferation of cells of the host following an initial graft-versus-host reaction. It is again suggested that this granulocytic response is a tissue-specific growth reaction resulting from the liberation of cell products in necrotic foci created in the initial immune reaction.

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